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RESEARCH PAPER

Supply of sulphur to S-deficient young barley seedlings restores their capability to cope with iron shortage

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Abstract

The effect of the S nutritional status on a plant's capability to cope with Fe shortage was studied in solution cultivation experiments in barley (*Hordeum vulgare* L. cv. Europa). Barley is a Strategy II plant and responds to Fe deficiency by secretion of chelating compounds, phytosiderophores (PS). All PS are derived from nicotianamine whose precursor is methionine. This suggests that a long-term supply of an inadequate amount of S could reduce a plant's capability to respond to Fe deficiency by limiting the rate of PS biosynthesis. The responses of barley (*Hordeum vulgare* L. cv. Europa) plants grown for 12 d on Fe-free nutrient solutions (NS) containing 0 or 1.2 mM SO_4^{2-}, was examined after 24 h or 48 h from transfer to NS containing 1.2 mM SO_4^{2-}. After the supply of S was restored to S-deprived plants, an increase in PS release in root exudates was evident after 24 h of growth in S-sufficient NS and the increment reached values up to 4-fold higher than the control 48 h after S resupply. When S was supplied to S-deficient plants, leaf ATPS (EC 2.7.7.4) and OASTL (EC 4.2.99.8) activities exhibited a progressive recovery. Furthermore, root *HvST1* transcript abundance remained high for 48 h following S resupply and a significant increase in the level of root *HvYS1* transcripts was also found after only 24 h of S resupply. Data support the idea that the extent to which the plant is able to cope with Fe starvation is strongly associated with its S nutritional status. In particular, our results are indicative that barley plants fully recover their capability to cope with Fe shortage after the supply of S is restored to S-deficient plants.

Key words: ATPS, OASTL, iron, methionine, phytosiderophores, sulphur.

Introduction

Over recent years, S deficiency has become widespread in many regions in the world. The occurrence of S deficiency has been described in cereals as well as in other crops and the reasons behind this trend are, mainly, the strong decrease in the inputs of S from atmospheric deposition and the use of high-analysis low-S fertilizers (Zhao *et al.*, 1999).

Although it is often reported that cereals have a relatively low requirement for S, they can be adversely affected by S deficiency in the field as a result of its effects on growth, grain yield, and quality (Zhao *et al.*, 1999). Moreover, several reports relate the plant's capability to respond to Fe deficiency to its S nutritional status (Astolfi *et al.*, 2003, 2006*a*; Bouranis *et al.*, 2003).

Fe deficiency is one of the major agricultural problems leading to reduced crop yields. Fe is abundant in soils, but it is mainly present as insoluble Fe(III) precipitates and thus plant uptake and the utilization of Fe are often limited. Furthermore, plant uptake of Fe involves complex processes. There are clear differences in Fe acquisition mechanisms between Strategy I and Strategy II plants.

Abbreviations: APS, adenosine 5′-phosphosulphate; ATPS, ATP sulphurylase; BSA, bovine serum albumin; DTT, DL-dithiothreitol; EDTA, ethylene diamine tetra-acetic acid; s.e., standard error; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid); NS, nutrient solution; OASTL, *O*-acetylserine(thiol)lyase; PMSF, phenylmethylsulphonyl fluoride; PS, phytosiderophores; PVP, polyvinylpyrrolidone.

Strategy II plants, like the graminaceous monocot barley (*Hordeum vulgare* L.), respond to Fe deficiency by the secretion of chelating compounds, phytosiderophores (PS), and by the uptake of Fe–PS complexes. PS are derived from nicotianamine whose precursors are methionine and SAM (Mori and Nishizawa, 1987). Recent evidence reasonably suggests that a limited supply of S could reduce plant capability to respond to Fe deficiency by limiting the rate of PS biosynthesis: in severely S-deficient plants the availability of compounds containing reduced S is lowered, thereby the synthesis of methionine decreases and, in turn, could limit the rate of PS biosynthesis.

Previously, it has been shown that the availability of sulphate in an Fe-deficient growth medium can affect the accumulation of PS and the incorporation of ^{14}C glucose into PS in Fe-deficient barley roots (Kuwajima and Kawai, 1997). Furthermore, there is direct evidence that low S availability can affect the response to Fe deficiency in barley at multiple control levels, influencing either the release of PS or the capability to take up Fe from the external solution (Astolfi *et al.*, 2006*a*). These results suggest that the requirement of S may be higher when plants are under Fe deficiency and, on the other hand, that the plant's response to Fe deficiency is modified by S supply.

The aim of the present experiments was to investigate, in young barley seedlings, whether the capability of coping with Fe-deficiency stress is reversible after the resupply of S to S-deficient plants, as well as the time-course of this reactivation. The rate of PS release, chlorophyll content, as well as activities of ATPS and OASTL, were examined. Furthermore, this study was set out to evaluate the expression pattern of a high affinity sulphate transporter (*HvST1*) and of an Fe–PS transporter (*HvYS1*) in barley roots.

Materials and methods

Growing conditions

Barley (*Hordeum vulgare* L. cv. Europa) seeds were germinated on moistened paper in the dark at 20 °C for 3 d. Seedlings were then transferred to plastic pots (12 seedlings per pot) filled with 2.2 l of continuously aerated Fe-free nutrient solutions (NS) (Zhang *et al.*, 1991) with or without 1.2 mM sulphate. The S-deficient NS was prepared by replacing sulphate salts (K^+, Mn^{2+}, Zn^{2+}, Cu^{2+}) with appropriate amounts of chloride salts (K^+, Mn^{2+}, Zn^{2+}, Cu^{2+}). Plants were grown in a climate chamber under 200 μmol m^{-2} s^{-1} PAR at leaf level and a 14/10 h day/night regime (temperature 27 °C diurnal; 20 °C nocturnal; relative humidity 80%). After 12 d from sowing, the supply of S (1.2 mM SO_4^{2-}) was restored to S-deprived plants for time periods varying from 24 h to 48 h.

In some experiments, plants were grown or resupplied with 100 μM Fe(III)-EDTA.

Collection of root exudates and determination of PS release

PS release from S-deficient and S-resupplied barley plants was analysed by determining the PS content in root washings. Barley plants were removed from the NS 2 h after the onset of the light period and the roots were washed twice for 1 min in deionized water. Root systems were then submerged in 500 ml deionized water for 3 h with continuous aeration. Thereafter, Micropur (10 mg l^{-1}) (Roth, Karlsruhe, Germany) was added to prevent microbial degradation of PS. PS content in the root washings were determined using the Fe-binding assay revised by Reichman and Parker (2006).

Methionine extraction and measurement

Methionine was determined as described in Kreft *et al.* (2003). Briefly, leaf and root tissues (about 100 mg per plant) were ground to a fine powder in liquid nitrogen in a bead mill and extracted three times for 20 min at 80 °C: once with 400 μl of 80% (v/v) aqueous ethanol (buffered with 2.5 mM HEPES-KOH, pH 7.5) and 10 μl of 20 μM L-nor-Val (as an internal standard), once with 400 μl of 50% (v/v) aqueous ethanol (buffered as before), and once with 200 μl of 80% (v/v) aqueous ethanol. Between the extraction steps, the samples were centrifuged for 10 min at 14 000 rpm, and the supernatants were collected. The combined ethanol/water extracts were stored at –20 °C or directly subjected to RP-HPLC using an ODS column (Hypersil C18; 150×4.6 mm i.d.; 3 μm; Knauer GmbH, Berlin) connected to an HPLC system (Dionex, Idstein, Germany). Methionine was measured by precolumn derivatization with OPA in combination with fluorescence detection (Lindroth and Mopper, 1979). Peak areas were integrated by using Chromeleon 6.30 software (Dionex) and subjected to quantification by means of calibration curves made from a standard mixture.

Enzyme extraction and assays

Frozen tissue (*c.* 1 g fresh weight) was ground to a fine powder in a prechilled mortar under liquid N_2. Cold extraction buffer containing 50 mM HEPES-KOH (pH 7. 4), 5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, 5 mM DTT, 1 mM PMSF, and 1% (w/v) PVP was added in a ratio of 1:7 (w/v). The brei was filtered through four layers of cheesecloth and the homogenate was centrifuged at 1000 *g* for 5 min at 4 °C. The resulting supernatant was desalted at 4 °C on a Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden) pre-equilibrated with extraction buffer minus Triton X-100. The desalted extract was then centrifuged at 30 000 *g* for 5 min at 4 °C. The supernatant was divided into 300 μl aliquots which were then frozen in liquid N_2 and stored at –80 °C until analysis.

ATP sulphurylase (ATPS; EC 2.7.7.4) activity was assayed by the bioluminescence technique (Schmutz and Brunold, 1982). ATP production during the enzyme reaction is coupled to the light-producing reaction catalysed by firefly luciferase (EC 1.13.12.7). The reaction mixture contained in a total volume of 0.25 ml: 16 mM TRIS-acetate buffer pH 7.75, 8 μM APS, 68 μM $Na_4P_2O_7$, 40 μl of firefly luciferase (ATP Monitoring Reagent, ThermoLab-Systems), and 5 μl of sample. Light emission was measured with an LKB 1250 luminometer. *O*-acetylserine(thiol)lyase (OASTL; EC 4.2.99.8) was determined following the procedures described by Ferretti *et al.* (1993).

Total RNA extraction and RT-PCR analysis

Isolation of total RNA from the roots of barley plants was performed using the Trizol® reagent system according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). One μg of DNase-treated RNA was reverse-transcribed by M-MLV (H-) Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) to synthesize the first strand of cDNA. One microlitre of the oligo$(dT)_{12}$-primed first-strand cDNA samples was used for PCR using reverse and forward primers designed on the basis of the published *HvST1* gene sequence (accession no. Q43482), as described in Astolfi *et al.* (2006*b*). Primers from Murata *et al.* (2006) were used specifically to amplify *HvYS1* (accession no. AB214183) cDNA. The level of the 18S gene expression was used as a control for quantification.

PCR conditions were: one initial cycle of 94 °C for 5 min, followed by cycles of 93 °C and 55 °C for 1 min each and 72 °C for 2 min for each cycle, with 40 and 30 cycles for *HvST1* and *18S* (control), respectively, all followed by a final extension time of 72 °C for 7 min. The number of PCR cycles was adjusted to obtain detectable amounts of amplicons without reaching signal saturation, which was accomplished.

The PCR program for *HvYS1* was one initial cycle of 94 °C for 5 min, followed by 32 cycles of 94 °C and 60 °C for 1 min each and 72 °C for 45 s for each cycle, plus a final extension time of 72 °C for 7 min.

All semi-quantitative RT-PCRs were performed in duplicate. RT-PCR amplification products were separated electrophoretically on 1% (w/v) agarose gels and stained with ethidium bromide.

Other measurements and statistics

The concentration of chlorophyll content per unit area was estimated in attached leaves by a SPAD portable apparatus (Minolta Co., Osaka, Japan) using the first fully expanded leaf from the top of the plant.

Protein content was determined according to Bradford (1976) using BSA as standard.

Each reported value represents the mean ±SD of measurements carried out in triplicate and obtained from four independent experiments. Statistical analyses of data were carried out by ANOVA tests with the GraphPad InStat Program (version 3.06). Significant differences were established by *post hoc* comparisons (HSD Tukey's test) at $P < 0.01$ or $P < 0.05$.

Results

Fe deprivation caused a significant decrease in shoot fresh weight of S-sufficient plants (inserts in Fig. 1). The biomass accumulation of Fe-deprived barley plants grown without added S (–S–Fe) was significantly lower than that of plants adequately fed with S (+S–Fe) (inserts in Fig. 1), the greatest effect being observed in shoots.

The chlorophyll content was determined by chlorophyll meter readings (SPAD) that provide a sensitive and accurate index of plant response to the Fe treatment and relative data are showed in Fig. 2. With regard to Fe deficiency, plants cultured without Fe displayed severe leaf chlorosis at harvest, with a 50% decrease in SPAD readings (insert in Fig. 2). Leaf chlorophyll content of Fe-deficient plants was slightly but significantly reduced by S-deprivation (insert in Fig. 2). Transferring the –S–Fe plants to S-sufficient NS significantly affected shoot, but not root growth rate or leaf chlorosis (Figs 1, 2). However, evident leaf regreening was observed by concomitant supply of both S and Fe to deficient plants, resulting in a SPAD units increase from 30% to 45% of the controls, after 24 h and 48 h from the beginning of S and Fe resupply, respectively (Fig. 2).

The effect of S availability status on PS release by Fe-deficient plants was compared next. This activity was measured 2 h after the onset of the light period, i.e. when barley shows a distinct peak in PS production (Marschner *et al.*, 1986). PSs were only detectable in Fe-deficient plants. The rate of PS release was markedly decreased by S deficiency to 25% of the rate recorded in plants grown in +S–Fe NS (Fig. 3). Resupply of S to S-deprived plants for 48 h, restored PS release that reached rates even higher than those of S-sufficient (Fe-deficient) plants (Fig. 3). In particular, an increase in PS release in root exudates was evident after 24 h of growth in S-containing NS, but the increment reached values up to 4-fold higher than in S-deficient (and Fe-deficient) plants after 48 h from S resupply (Fig. 4); on the other hand, in plants that were supplied with both S and Fe, PS release remained at very low levels (Fig. 4).

The evidence that PS are derived from nicotianamine, whose precursors are methionine and SAM, might reasonably suggest that the requirement of reduced S for methionine biosynthesis may be higher when plants are under Fe deficiency conditions and release PS at high rates. To this end, the change in the level of this S-containing amino acid was measured (Fig. 5). In +S plants, the methionine content was almost unchanged by Fe-deficiency in leaves, but in the root tissue was 75% higher in

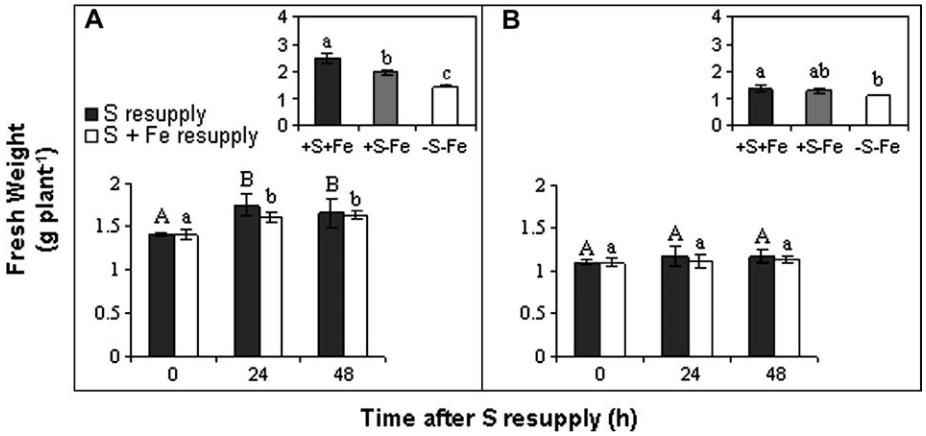

Fig. 1. Shoot (A) and root (B) fresh weight of Fe-deficient barley plants. Plants were grown on S-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} for 24 or 48 h. (Inserts) Response to S deficiency of barley shoots (a) and roots (b) grown on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 (–S–Fe) or 1.2 mM SO_4^{2-} (+S–Fe). Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters ($P < 0.05$) ($n=4$).

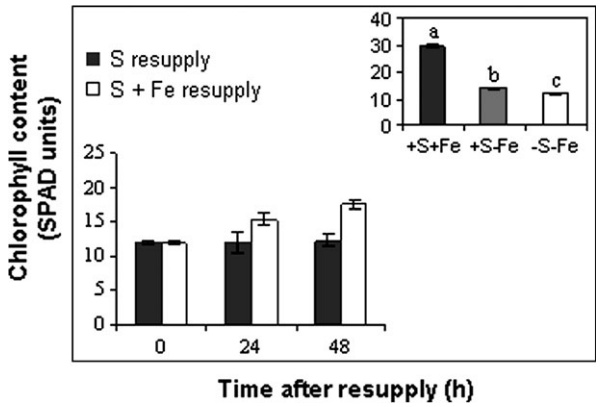

Fig. 2. Chlorophyll content of Fe-deficient barley plants. Plants were grown on S-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} (black bars) or 1.2 mM SO_4^{2-}+100 μM Fe(III)-EDTA (white bars) for 24 h or 48 h. (Insert) Effect of S deficiency on leaf chlorophyll content of barley plants grown on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 mM (–S–Fe) or 1.2 mM SO_4^{2-} (+S–Fe). SPAD readings were made using the first fully expanded leaf from the top of the plant. Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters (P <0.01) (n=4).

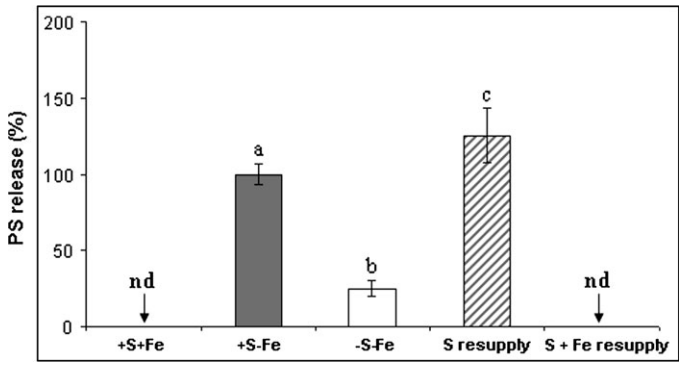

Fig. 3. PSs release by Fe-deficient barley plants grown for 12 d on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 (–S–Fe) (white bar) or 1.2 mM SO_4^{2-} (+S–Fe) (black bar), and by S (striped bar) or (S+Fe) resupplied plants for 48 h. Data are expressed as percentage in respect to the S-sufficient (–Fe) control (100%). Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters (P <0.05) (n=4).

–Fe plants than in those being Fe-sufficient (inserts in Fig. 5). Furthermore, when S and Fe were completely omitted from NS, a further increase of methionine content was found at both shoot and root level (inserts in Fig. 5). Changes of methionine content were next measured in leaves and roots after supplying S or (S+Fe) to deficient barley plants. Methionine content increased sharply in both shoots and roots reaching levels nearly 2-fold higher than those in S-deficient plants at 24 h from S resupply (Fig. 5). Also (S+Fe) resupply resulted in a transient increase in

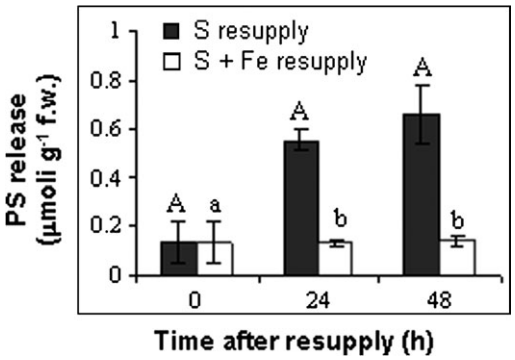

Fig. 4. PSs release by Fe-deficient barley plants. Plants were grown on S-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} (black bars) or 1.2 mM SO_4^{2-}+100 μM Fe(III)-EDTA (white bars) for 24 h or 48 h. PSs release was measured by determining the PSs content in root washings. Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters (P <0.01) (n=4).

tissue methionine content, although, at least for the roots, to a lesser extent than when S alone was resupplied (Fig. 5). In both trials, the amino acid content declined prolonging the S treatment.

Since methionine biosynthesis involves the S assimilation pathway through a trans-sulphuration reaction from cysteine to *O*-phosphohomoserine (Hesse and Hoefgen, 2003), changes in ATPS and OASTL activities (the first and the last enzymes of the S assimilation pathway, respectively) have been investigated further. Results showed that Fe deficiency significantly increased leaf ATPS activity (+60%), but did not significantly affect root ATPS activity in +S plants (inserts in Fig. 6). On the other hand, growing Fe-deficient plants without added S significantly stimulated root ATPS activity (+143% of the +S control), however, no increase of this enzyme activity has been observed at leaf level (inserts in Fig. 6). On the other hand, OASTL activity in both roots and leaves was almost unaffected when +S plants were subjected to Fe deficiency, but was significantly increased in both roots and leaves when Fe-deficient plants were subjected to S deficiency; the percentage of stimulation varying from 15% to 30% of the +S controls (inserts in Fig. 7). When S was supplied to deficient plants, leaf ATPS activity exhibited a progressive recovery until it reached levels 4-fold higher than those in leaves of control plants at 48 h from S resupply (Fig. 6A); a recovery was also observed when the Fe- and S-deficient plants were supplied with both S and Fe, but its maximum increase did not exceed 170%. Root ATPS activity was essentially unaffected by the resupply of S alone, while being slightly decreased by a concomitant resupply of S and Fe (Fig. 6B).

A recovery was also seen for leaf OASTL activity after the supply of S to deficient plants, although to a lesser extent than ATPS (activity increased from 53% to 60% of control after 24 h and 48 h from S resupply, respectively) (Fig. 7A). Supply of both S and Fe left OASTL activity in the leaves almost unchanged. Root OASTL activity

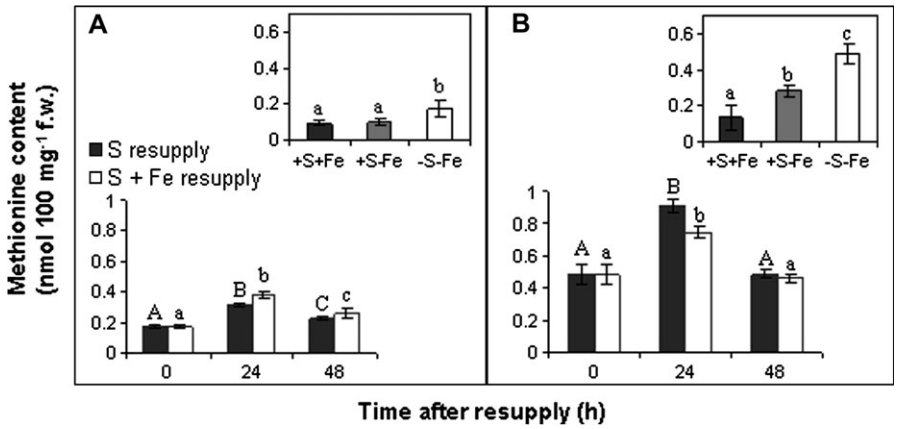

Fig. 5. Methionine content of Fe-deficient barley shoot (A) and root (B). Plants were grown on S-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} (black bars) or 1.2 mM SO_4^{2-}+100 μM Fe(III)-EDTA (white bars) for 24 h or 48 h. (Inserts) Response to S deficiency of barley shoots (a) and roots (b) grown on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 (–S–Fe) or 1.2 mM SO_4^{2-} (+S–Fe). Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters (P <0.01) (n=4).

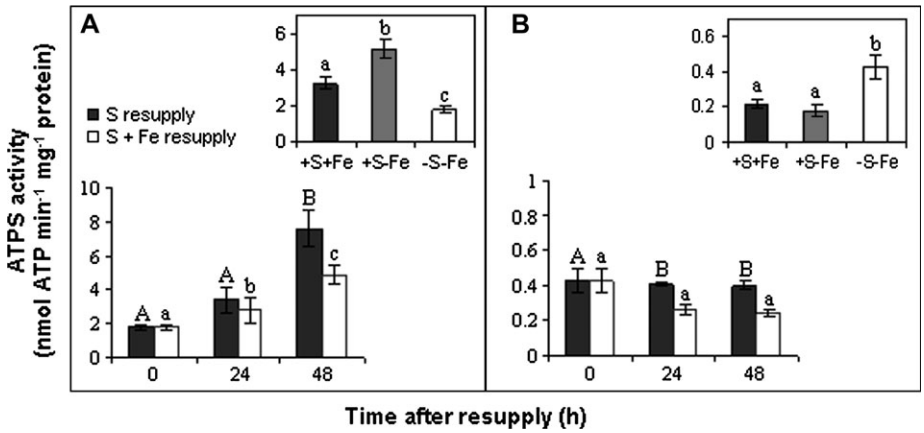

Fig. 6. Changes in ATPS activity (nmol ATP min^{-1} mg^{-1} protein) in leaves (A) and roots (B) of Fe-deficient barley plants. Plants were grown on S-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} (black bars) or 1.2 mM SO_4^{2-}+100 μM Fe(III)-EDTA (white bars) for 24 h or 48 h. (Inserts) Effect of S deficiency on leaf and root ATPS activity of barley plants grown on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 (–S–Fe) or 1.2 mM SO_4^{2-} (+S–Fe). Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters (P <0.05) (n=4).

followed a pattern similar to that of ATPS activity, being almost unaffected by the resupply of S alone, and slightly decreased by (S+Fe) resupply (Fig. 7B).

A higher transcript abundance of the *HvST1* gene, coding for a high affinity sulphate transporter, was observed in roots of S-starved barley plants, in accordance with other reports (Hawkesford *et al.*, 1995; Smith *et al.*, 1997; Vidmar *et al.*, 1999; Astolfi *et al.*, 2006*b*) (Fig. 8A). Interestingly, the estimation of changes in transcript levels revealed that no down-regulation of *HvST1* gene expression was observed in barley roots up to 48 h after supplying S or (S+Fe) to deficient plants (Fig. 8B, C).

The gene *HvYS1* is assumed to code for a specific Fe(III)-PS transporter in barley roots (Murata *et al.*, 2006). In accordance with work by Murata *et al.* (2006), its expression was induced in roots of plants that had been subjected to 12 d of Fe starvation (Fig. 8A). A substantial increase in gene expression was observed after the supply of S was restored to S-deprived plants (Fig. 8B). On the other hand, *HvYS1* transcript abundance was progressively decreased when the S- and Fe-deficient plants were supplied with both nutrients (Fig. 8C).

Discussion

Experimental evidence has indicated that significant interactions between plant Fe acquisition mechanisms and external sulphate supply can occur. For example, it has been demonstrated that S-deficient maize plants exhibited a lower leaf Fe content than S-sufficient ones (Astolfi *et al.*, 2003). In addition, it was shown that the availability of SO_4^{2-} could affect accumulation (Kuwajima and Kawai, 1997) and release (Astolfi *et al.*, 2006*a*) of PS in Fe-deficient

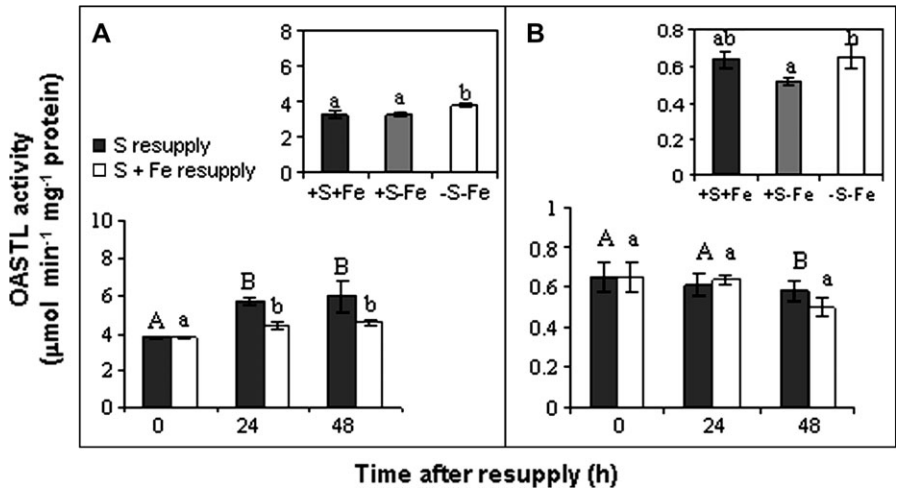

Fig. 7. Changes in OASTL activity (μmol min^{-1} mg^{-1} protein) in leaves (A) and roots (B) of Fe-deficient barley plants. Plants were grown on S-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} (black bars) or 1.2 mM SO_4^{2-}+100 μM Fe(III)-EDTA (white bars) for 24 h or 48 h. (Inserts) Effect of S deficiency on leaf and root OASTL activity of barley plants grown on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 (–S–Fe) or 1.2 mM SO_4^{2-} (+S–Fe). Data are means ±SD of four independent replications run in triplicate. Significant differences between samples are indicated by different letters (P <0.05) (n=4).

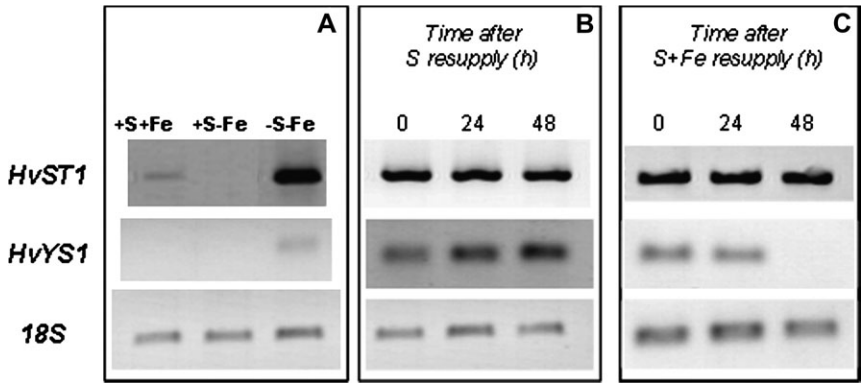

Fig. 8. RT-PCR expression analysis of a high affinity sulphate transporter (*HvST1*) and of an iron-phytosiderophore transporter (*HvYS1*) in roots of barley plants. Plants were grown on complete NS containing 1.2 mM SO_4^{2-} and 100 μM Fe(III)-EDTA (+S+Fe) or on Fe-free NS containing 0 (–S–Fe) or 1.2 mM SO_4^{2-} (+S–Fe) (A) or on S-and Fe-free NS for 12 d, followed by a transfer to NS containing 1.2 mM SO_4^{2-} (S resupply) (B) or 1.2 mM SO_4^{2-}+100 μM Fe(III)-EDTA (S+Fe resupply) (C) for 24 or 48 h. A 18S PCR product was used as a cDNA calibration control.

barley roots, possibly impairing the capacity of these plants to cope with Fe-deficiency (Romheld, 1987; Marschner and Römheld, 1994). It is generally accepted that enhanced release of PS from roots of graminaceous plants is a typical response to Fe deficiency (Romheld, 1987; Marschner and Romheld, 1994). When Fe-deficient plants were grown in S-free NS, PS release rate was four times lower than in control S-sufficient plants (Fig. 3). In the present work it was shown that the resupply of S to Fe- and S-deprived plants could restore their capacity to release PS within 24 h (Fig. 4). All PS are derived from nicotianamine, whose precursors are methionine and SAM (Mori and Nishizawa, 1987). Therefore, it is likely that PS release is limited because there is not an adequate amount of methionine or SAM, probably due to low S availability. This idea is consistent with the observed greater accumulation of methionine in barley shoots and roots when Fe- and S-deficient plants were transferred to a S-containing nutrient solution (Fig. 5). Such an overshoot has also been shown for *Arabidopsis* when resupplying sulphate after S-starvation (data not shown). The increase at root level is particularly important since PS are synthesized in the roots and the methionine for their synthesis is not translocated from the shoot to the roots (Nakanishi *et al.*, 1999). It was previously shown that Fe deficiency affected the partitioning of the reduced S pool, inducing the translocation of thiols from the shoots to the roots and the accumulation of cysteine in the roots, possibly to sustain PS synthesis (Astolfi *et al.*, 2006*b*). However, since some authors found that with S-starvation (in *Arabidopsis*), methionine levels remained fairly constant long into the starvation, but that SAM was drastically reduced (Nikiforova *et al.*, 2005), the

possibility that the drastic reduction of SAM rather than methionine availability might be the reason for reduced PS levels cannot be ruled out. The suggestion that SAM declined under S-starvation seems to be confirmed by the concomitant reduction in chlorophyll content and associated leaf chlorosis in S-starved plants (data not shown; Astolfi *et al.*, 2006*a*). In the same study it was speculated that leaf chlorosis might be due to SAM reduction with an effect on chlorophyll biosynthesis as well (Nikiforova *et al.*, 2005).

Therefore, it can be seen that the greatest effect of S resupply was expressed as a better recovery of the plant's capability to release PS. High rates of PS production seem to depend on the methionine content, which in turn depends on the S assimilation pathway, the role of which is to contribute to the supply of cysteine which acts as a donor of reduced S in the synthesis of methionine. Thus, experiments were carried out to evaluate changes in ATPS and OASTL activity, the first and the last enzymes of the S assimilation pathway, respectively. When S was supplied to S-deficient plants, leaf activity of both enzymes exhibited a progressive recovery (Figs 6, 7). In particular, OASTL activity increased from 53% to 60% of control, after 24 h and 48 h from S resupply, respectively, while ATPS activity recovered to a greater extent, reaching values 4-fold higher than the control after 48 h from the beginning of S resupply. The extraordinary recovery of ATPS activity in barley leaves upon S resupply indicates an increase of S assimilation, which is realistically explained by assuming an increased demand of reduced S being forwarded to the roots for methionine and SAM biosynthesis and, consequently, PS synthesis. This finding is indeed in contrast to what is widely reported when S-deficient plants are resupplied with S. It is well known that S starvation results in a strong increase of S uptake and ATPS activity (Smith *et al.*, 1997; Lappartient *et al.*, 1999), but when plants are resupplied with S, ATPS activity quickly returns to the normal level (Brunold *et al.*, 1987; Hawkesford *et al.*, 1995; Lappartient and Touraine, 1996). Although OASTL activity is usually reported not to be univocally affected by external sulphate supply (Takahashi and Saito, 1996; Kopriva and Rennenberg, 2004), these observations indicate an increase of leaf OASTL enzyme activity upon S deprivation (Astolfi *et al.*, 2006*b*) and following S resupply.

It seems, however, that the response of barley plants to S resupply does not involve the enhanced assimilation of sulphate at root level since the activity of the two enzymes (ATPS and OASTL) was not significantly modified by the treatment (Figs 6, 7).

Barley plants are apparently responding to the Fe deficiency stress by inducing the enzymes involved in the uptake and assimilation of sulphate, probably due to a higher demand for cysteine. This idea is supported by gene expression analysis of a high affinity sulphate transporter (*HvST1*) in barley roots. It has been reported that the expression of high-affinity sulphate transporters is increased upon S starvation (Hawkesford *et al.*, 1995; Smith *et al.*, 1997; Vidmar *et al.*, 1999; Astolfi *et al*,. 2006*b*), but it is decreased within a few hours after S resupply (Smith *et al.*, 1997; Bolchi *et al.*, 1999; Lappartient *et al.*, 1999; Vidmar *et al.*, 1999; Takahashi *et al.*, 2000). In our experiments, the expression level of the *HvST1* gene was not altered when the S- and Fe-deficient plants were resupplied with S, as no reduction in the level of root *HvST1* transcripts was observed after S resupply (Fig. 8). As already pointed out, a concomitantly enhanced assimilation activity and increased *HvST1* gene expression indicated that S incorporation into compounds containing reduced-S was compensated by augmented S-uptake to support PS production. Furthermore, while further studies are needed, the *HvST1* expression pattern would be consistent with a function in S acquisition activated by Fe deficiency in barley roots, indicating that the transcript levels of *HvST1* could, in addition, be regulated by signals other than the S nutritional status of the plants.

To address the question of whether an adequate plant S nutritional status not only promotes the plant's ability to synthesize PS, but also contributes to improve Fe uptake, the expression pattern of a gene encoding for a Fe-PS transporter (*HvYS1*) in barley roots has been examined. *HvYS1* is only expressed in roots and has high substrate specificity: only the Fe(III)–PS complex could be transported by *HvYS1* (Murata *et al.*, 2006). A significant increase in the level of root *HvYS1* transcripts was found after 24 h of S resupply (Fig. 8). The finding that *HvYS1* expression changes in response to S external supply suggests that, as soon as the plants sensed changes in S availability they try to increase Fe acquisition from the medium, thus enhancing both PS synthesis and Fe(III)–PS complex uptake. This hypothesis is in good agreement with the fast recovery of chlorophyll concentration in leaves of plants that were resupplied with both S and Fe (Fig. 2). On the other hand, *HvYS1* expression was reduced by supplying Fe and S to deficient plants, a result that is consistent with the low PS release observed in these plants (Fig. 4).

In conclusion, results of the present work extend our previous finding on the interactions between S and Fe nutrition. Data support the idea that barley plants with limited S supply are more susceptible to Fe chlorosis and, on the other hand, that the extent to which the plant is able to cope with Fe starvation is strongly dependent on its S nutritional status.

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References

Astolfi S, Cesco S, Zuchi S, Neumann G, Roemheld V. 2006*a*. Sulphur starvation reduces phytosiderophores release by Fe-deficient barley plants. *Soil Science and Plant Nutrition* **52,** 80–85.

Astolfi S, Zuchi S, Cesco S, Sanità di Toppi L, Pirazzi D, Badiani M, Varanini Z, Pinton R. 2006*b*. Fe deficiency induces

sulphate uptake and modulates redistribution of reduced sulphur pool in barley plants. *Functional Plant Biology* **33,** 1055–1061.

Astolfi S, Zuchi S, Passera C, Cesco S. 2003. Does the sulphur assimilation pathway play a role in the response to Fe deficiency in maize (*Zea mays* L.) plants? *Journal of Plant Nutrition* **26,** 2111–2121.

Bolchi A, Petrucco S, Tenca PL, Foroni C, Ottonello S. 1999. Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by l-cysteine. *Plant Molecular Biology* **39,** 527–537.

Bouranis DL, Chorianopoulou SN, Protonotarios VE, Siyannis VF, Hopkins L, Hawkesford MJ. 2003. Leaf response of young iron-inefficient maize plants to sulfur deprivation. *Journal of Plant Nutrition* **26,** 1189–1202.

Bradford MM. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Brunold C, Suter M, Lavanchy P. 1987. Effect of high and low sulfate concentrations on adenosine 59-phosphosulfate sulfotransferase from *Lemna minor* L. *Physiologia Plantarum* **70,** 168–174.

Ferretti M, Ghisi R, Merlo L, Dalla Vecchia F, Passera C. 1993. Effect of cadmium on photosyntetic sulphate and nitrate assimilation. *Photosyntetica* **29,** 49–54.

Hawkesford MJ, Schneider A, Belcher AR, Clarkson DT. 1995. Regulation of enzymes involved in the sulphur assimilatory pathway. *Zeitschrift für Pflanzenernährung und Bodenkunde* **158,** 55–57.

Hesse H, Höfgen R. 2003. Molecular aspects of methionine biosynthesis in Arabidopsis and potato. *Trends in Plant Science* **8,** 259–262.

Kopriva S, Rennenberg H. 2004. Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. *Journal of Experimental Botany* **55,** 1831–1842.

Kreft O, Hoefgen R, Hesse H. 2003. Functional analysis of cystathionine gamma-synthase in genetically engineered potato plants. *Plant Physiology* **131,** 1843–1854.

Kuwajima K, Kawai S. 1997. Relationship between sulfur metabolism and biosynthesis of phytosiderophores in barley roots. In: Ando T, Fujita K, Mae T, Matsumoto H, Mori S, Sekiya J, eds. *Plant nutrition: for sustainable food production and environment*. The Netherlands: Kluwer Academic Publishers, 285–286.

Lappartient AG, Touraine B. 1996. Demand-driven control of root ATP sulphurylase activity and SO_4^{2-} uptake in intact canola. *Plant Physiology* **111,** 147–157.

Lappartient AG, Vidmar JJ, Leustek T, Glass ADM, Touraine B. 1999. Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. *The Plant Journal* **18,** 89–95.

Lindroth P, Mopper K. 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatisation with *o*-phthaldialdehyde. *Analytical Chemistry* **51,** 1667–1674.

Marschner H, Römheld V. 1994. Strategies of plant acquisition of iron. *Plant and Soil* **165,** 261–274.

Marschner H, Römheld V, Kissel M. 1986. Different strategies in higher plants in mobilization and uptake of iron. *Journal of Plant Nutrition* **9,** 695–713.

Mori S, Nishizawa N. 1987. Methionine as a dominant precursor of phytosiderophores in Graminea plants. *Plant and Cell Physiology* **28,** 1081–1092.

Murata Y, Ma JF, Yamaji N, Ueno D, Nomoto K, Iwashita T. 2006. A specific transporter for iron(III)-phytosiderophore in barley roots. *The Plant Journal* **46,** 563–572.

Nakanishi H, Bughio N, Matsuhashi S, Ishioka NS, Uchida H, Tsuji A, Osa A, Sekine T, Kume T, Mori S. 1999. Visualizing real time [^{11}C] methionine translocation in Fe-sufficient and Fe-deficient barley using a Positron Emitting Tracer Imaging System (PETIS). *Journal of Experimental Botany* **50,** 637–643.

Nikiforova VJ, Kopka J, Tolstikov V, Fiehn O, Hopkins L, Hawkesford MJ, Hesse H, Hoefgen R. 2005. Systems rebalancing of metabolism in response to sulfur deprivation, as revealed by metabolome analysis of Arabidopsis plants. *Plant Physiology* **138,** 304–318.

Reichman SM, Parker DR. 2006. Critical evaluation of three indirect assays for quantifying phytosiderophores released by the roots of Poaceae. *European Journal of Soil Science* **28,** 844–853.

Römheld V. 1987. Different strategies for iron acquisition in higher plants. *Physiologia Plantarum* **70,** 231–234.

Schmutz D, Brunold C. 1982. Rapid and simple measurement of ATP sulphurylase activity in crude plant extracts using an ATP meter for bioluminescence determination. *Analytical Biochemistry* **121,** 151–155.

Smith FW, Hawkesford MJ, Ealing PM, Clarkson DT, Vanden Berg PJ, Belcher AR, Warrilow AGS. 1997. Regulation of expression of a cDNA from barley roots encoding a high affinity sulphate transporter. *The Plant Journal* **12,** 875–884.

Takahashi H, Saito K. 1996. Subcellular localization of spinach cysteine synthase isoforms and regulation of their gene expression by nitrogen and sulfur. *Plant Physiology* **112,** 273–280.

Takahashi H, Watanabe-Takahashi A, Smith FW, Blake-Kalff M, Hawkesford MJ, Saito K. 2000. The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana. The Plant Journal* **23,** 171–182.

Vidmar JJ, Schjoerring JK, Touraine B, Glass ADM. 1999. Regulation of the *HvST1* gene encoding a high affinity sulfate transporter from. *Hordeum vulgare. Plant Molecular Biology* **40,** 883–892.

Zhang FS, Römheld V, Marschner H. 1991. Role of the root apoplasm for iron acquisition by wheat plants. *Plant Physiology* **97,** 1302–1305.

Zhao FJ, Hawkesford MJ, McGrath SP. 1999. Sulphur assimilation and effects on yield and quality of wheat. *Journal of Cereal Science* **30,** 1–17.